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(57) Abstract

There are disclosed pharmaceuticals which comprise either part or all of a polypeptide or a nucleotide sequence coding for said part or all of a polypeptide which polypeptide is substantially homologous to a mutant protein produced in consequence of its mutation in a gene sequence which shifts translation from the gene sequence out of the list reading enzyme into either the second or third reading frames downstream of the mutation and which said part or all of a polypeptide is able to involve an immune response against the mutant protein.

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IMMUNOGENIC PHARMACEUTICALS

The present invention concerns materials and methods relating to the preparation of immunogenic pharmaceuticals.

It has been suggested that antigens in malignant cells may be used as vaccines to induce tumour-specific cell-mediated immunity (Pardoll, D.W., Nature 369, 357-358 (1994)) and work has focussed on various sources of antigens, including viral (Papadopoulos, E.B., et al., N.Engl. J. Med. 330, 1185-1191(1994); Feitkamp, M.C.W., et al., Eur. J. Immun. 23, 2242-2249 (1993), fetal and tissue-specific antigens (Pardoll, D.W., 1994 supra.), and point mutations (Peace, D.J., et al., J. Exp. Med. 179, 473-479 (1994).

The present application newly teaches that antigens may arise by frameshift mutations which give rise to new segments of unique protein. Antigens deriving from new segments of unique protein in consequence of a frameshift mutation may be a previously unrecognized source of tumour-specific antigens or antigens specific to any other disease state associated with a frameshift mutation.

Research to date indicates that peptide fragments of intracellular proteins e.g. viral protein peptide fragments can be presented at the surface of somatic cells in association with MHC Class 1 molecules. The mechanism is known as antigen presentation. It is

thought that viral or host proteins are made on free ribosomes in the cytosol. These proteins are then fragmented by proteolytic enzymes and the peptide fragments transported by a signal-independent mechanism into a compartment of the host cell (probably the endoplasmic reticulum) where they bind to the MHC Class 1 molecules. This complex is transported to the cell surface and the peptide is displayed at the cell surface to circulating cytotoxic lymphocytes.

The mechanism allows the immune system to detect intracellular viral antigens early in infections. It is also responsible for the rejection of unrelated organs after transplantation. Although the mechanism probably evolved to deal with intracellular infectious agents, it is possible that this mechanism may be active in uninfected cells for the continual presentation of proteins of the cell, to the immune system for surveillance. For a Review of the above, see Townsend, A., "Presentation of Viral Antigens to the Immune System" Les Cahiers de la Foundation Louis Jeantet dé Medecine 1992 No. 7.

On the assembly of the complex of MHC molecules with peptide fragments, it is known that virus peptides appear to aid assembly and stabilisation of the MHC complex (Townsend, A., et al (1989) Nature Vol 340, No. 6233 pp443-448 and Elvin, J., et al (1993) J. Immunol Methods 158 p161-171).

The ability of an MHC molecule to form complexes

with peptides has been utilised to identify a potentially immunogenic sequence defined from antigens. Immunogenic peptide sequences will be recognised by cells of patients previously infected with the pathogen from which the peptide sequence derived. This approach has been exemplified by the binding of a peptide from HIV-1 gag with MHC (Elvin, J., et al 1993 supra).

In certain disease conditions (e.g. cancer), a normal (meaning 'healthy') cell mutates. The normal version of the cell will contain a particular gene sequence which encodes the corresponding normal protein. However, the mutation may comprise either the deletion of one or more bases from the normal gene sequence or the addition of one or more bases into the normal gene sequence. This can result in a shift of the reading frame.

Usually these sorts of frameshifting events lead to a truncated protein, because the 2nd and 3rd reading frames contain frequent STOP codons. However, between the frameshift mutation and the nearest downstream STOP codon the frameshifting event results in a protein sequence different to that of the corresponding normal protein. The present application teaches that mutant proteins produced as a result of frameshifting events and/or polypeptide/peptide fragments of these mutant proteins act as antigens, because they are not usually expressed in the mammalian body.

As mentioned above, it is thought that the proteins

of the cell are being continually sampled, digested and peptide fragments resulting from the digestion, presented at the cell surface as a complex with MHC molecules for inspection by circulating lymphocytes. Where the proteins are normal for the individual, the polypeptide/peptide fragments are recognised by the individual's lymphocytes as being native to the individual and no attack is mediated by the lymphocytes.

However the present application teaches that where a mutant protein is in consequence of a frameshift mutation, the mutant protein itself and at least some of the peptide/polypeptide fragments deriving therefrom will be new to the individual. In which case when these new fragments are presented in association with MHC to the circulating lymphocytes, they will be seen as foreign and the lymphocytes will mediate an immune response thereagainst. This response would be analogous to rejection of a foreign tissue graft. Therefore the present application teaches that a mutant protein resulting from a genetic mutation which shifts translation into the second and third reading frames and peptide/polypeptide fragments deriving from such a mutant protein may be of value as an active ingredient of a pharmaceutical to invoke an immune response against the mutant protein. Thus the new polypeptides and peptides may be of value in the formulation of vaccines.

Many colon cancers appear to be associated with frameshifting gene mutations (Fearon et al., Cell Vol. 61

p759-767, (1990); Nishisho. I., et al., Science 253, 665-669 (1991); Myoshi Y., et al., Hum. Molec. Genet., 1, 229-233 (1992)). Frameshift mutations also occur in gastric and pancreatic carcinoma (Horii A., et al., Cancer Res. 62, 6696-6698 (1992) and Horii A., et al., Cancer Res. 52, 3231-3233 (1992)) and in other malignancies (Harris C.C. et al., N.Engl. J. Med., 329, 1318-1327 (1993)).

In particular, the adenomatous polyposis coli (APC) gene from chromosome 5q21 has been suggested to contribute to colorectal tumorigenesis. The APC gene has been found to code an unusually large (300 KDa) protein and the gene has been sequenced and a predictive amino acid sequence disclosed (Kinzler et al., Science Vol. 253 p661,(1991)).

Somatic mutations of the APC gene have been identified as occurring in cases of patients with colorectal tumours (Myoshi et al., 1992 supra.). Myoshi et al. provide details of 43 cases of carcinoma of the colon. The present inventor has analysed these cases. About half the mutations form a premature STOP codon in the gene at the site of mutation. This results in a truncated version of the APC protein which to date appears to be of no practical immunological interest. However the remaining half (21) of mutations (small deletions or insertions of bases) result in frameshifts, and the translation of a mutant polypeptide sequence downstream of the mutation until a STOP codon is reached.

The frameshift mutations in these cases would give rise to new sequences of up to 56 amino acids, with a mean length of 16.1 residues.

The 11 cases with predicted new sequences of 16 amino acid residues or longer are listed in Table 1 below. Several of the new reading frames contain regions of sequence that are likely to bind common MHC Class 1 molecules (Falk K., et al., Nature 351, 290-294 (1991))

TABLE 1

NEW SEQUENCES GENERATED BY FRAMESHIFT MUTATIONS IN THE APC GENE

Codon	Mutation Size	New Sequence
298	2bp del	SSST/LCTSKADKSSGNQGGNGVFIVVNAWYS 27aa
540	1bp del	SEDL/TAGYCKCFEEFVLASRCK 18aa
1068	4bp del	EQRQ/GIKVQLILFILRALMINTSSSNHIL DSRNVFLHTGHGEPMVQKQIEWVLIMELIKM 56aa
1353	8bp del	HKAV/FRSEISLQKWCSDTQKST 18aa
1398	1bp del	DSFE/SVRLPAPFRVNHAVEW 16aa
1420	1bp del	IISP/VIFQIALDKPCHQAEVKHLHHLLK QLKPSEKYLKIKHLLLKRERVDLSKLQ 51aa
1439	1bp del	RSKT/LHHLLKQLKPSEKYLKIKHLL LKRERVDLSKLQ 33aa
1446	10bp del	PPQT/GEKYLKIKHLLLKRERVDLSKLQ 23aa
1488	1bp del	DADT/YYILPRKVLQMDFLVHPA 18aa
1490	1bp del	DTLL/LLPRKVLQMDFLVHPA 16aa
1493	11bp del	LHFA/SRWIFLFIQPECSEPR 16aa

The sequences underlined were found to stabilize the assembly of HLA A2 as described in Townsend A., et al. Cell 62, 285-295, 1990, the sequence in bold induced K^d restricted CTL in BALB/c mice.

The present inventor has confirmed that the sequences underlined in Table 1 above, have the ability to induce the assembly of HLA A2 Class I molecules in vitro.

Thus the present application teaches that until such time as any ribosome reaches a STOP codon, a new peptide/ polypeptide sequence is in fact translated between a frameshift mutation and the stop codon and that this new sequence and polypeptide and peptide fragments from it, will be disease (e.g. tumour)specific antigens which when presented at the cell surface in association with MHC will be seen as foreign.

Given that these new polypeptide and peptide sequences will be unique antigens specific to the disease associated with the frameshift, peptides/ polypeptides comprising part or all of the sequences of these unique antigens may be used to treat individuals in order to induce immune responses to mutant cellular proteins associated with the disease e.g. colon carcinoma.

Therefore potentially all of the translation products of the 2nd and 3rd reading frames of the APC gene could have potential as therapeutic and prophylactic vaccines.

Thus the present invention provides proteins and polypeptide and peptide fragments thereof, which proteins have substantially the sequence of corresponding mutant proteins which are produced in consequence of gene mutations associated with a disease state which shift translation into the second or third reading frames downstream of the gene mutation, and which proteins and polypeptide and peptide fragments thereof and mutant proteins are antigenic.

Such a mutant protein may be encoded by the adenomatous polyposis coli (APC) gene of chromosome 5q21.

The mutant protein may be encoded by the second reading frame lying between codons 1418 and 1472 of the APC gene.

The mutant protein may be encoded by the second reading frame lying between codons 1472 and 1506 of the APC gene.

The peptides may be selected from the sequences

KYLKIKHLL
QLKPSEKYL
ILYYILPRK
KVLQMDFLV

The present invention also provides nucleotide sequences coding for proteins and polypeptide and peptide fragments thereof and mutant proteins as described above.

The present invention also provides these nucleotide sequences as incorporated into vectors such as transfer, expression and vaccine vectors. The vaccine vector may be based upon vaccinia virus or other useful viral

vectors and Ty particles.

The present invention also provides recombinant host cells which contain the nucleotide sequence or vector.

The present invention also provides the proteins and polypeptide and peptide fragments in combination with one or more moieties to increase antigenicity. The combination may be by way of conjugation or admixture.

The present invention also provides pharmaceutical compositions which comprise either (i) a protein or polypeptide/peptide fragment thereof as described above; (ii) a nucleotide sequence as described above; or (iii) a vector as described above.

In particular, the pharmaceutical composition may be a therapeutic vaccine comprising a vector based upon virus (such as vaccinia virus) which incorporates a nucleotide sequence as described above. Alternatively the pharmaceutical composition may be a therapeutic vaccine comprising a vector based on a Ty particle which incorporates a nucleotide sequence as described above.

The vaccines may also be used prophylactically eg to protect against the development of secondary cancers.

The present invention also provides treatment methods using these pharmaceuticals/vaccines to treat a patient for a disease condition associated with a frameshift mutation.

The present invention also provides a method for identifying a mutant protein and polypeptide and peptide fragments thereof and which are as described above, the

method comprising the steps of:

(i) extracting DNA from a tissue sample derived from an individual with a disease associated with at least one gene mutation which shifts translation into the second or third reading frames downstream of the mutation;

(ii) selecting a section of DNA for amplification on the basis of an expectation of a mutation in that section;

(iii) amplifying the selected section of DNA:

(iv) sequencing part or all of the amplified DNA and selecting nucleotide sequences encoding amino acid sequences likely to bind MHC molecules and which are in the second or third reading frame as compared to a corresponding nucleotide sequence in the first reading frame;

(v) testing the antigenicity of the polypeptide/peptide sequences encoded by the nucleotide sequences selected in step (iv).

In the alternative, the method may comprise the steps of

(a) extracting DNA from a tissue sample derived from an individual with a disease associated with at least one gene mutation which shifts translation into the second or third reading frames downstream of the mutation;

(b) selecting a section of DNA for amplification on the basis of an expectation of a mutation in that

section;

(c) amplifying the selected section of DNA;

(d) making an expression vector containing the selected section of DNA, transforming a suitable host cell and expressing said selected DNA;

(e) testing the expression product for binding to and stabilisation of an MHC molecule derived from a said individual; and

(f) testing the antigenicity of an expression product which binds and stabilises a said MHC molecule.

The two methodologies may be combined. Thus part or all of the nucleotide sequences selected in step (iv) on the basis of their encoding amino acid sequences likely to bind MHC molecules and which are in the second or third reading frame may be incorporated into expression vectors and expressed as in step (d). The expression product will be tested as in steps (e) and (f).

The therapeutic utility of any proteins/polypeptides/ peptides may be tested in accordance with standard procedures. Likewise any proteins/polypeptides/peptides which as a result of such tests are thought to have therapeutic utility may be both formulated into pharmaceuticals and vaccines and further tested both in vitro and in vivo (animal and human studies) in accordance with techniques and procedures commonly used and well-known in the art.

This new idea generally outlined above will now be discussed by way of example only, and not by way of

limitation with reference to mutant polypeptide/peptide sequences derived from the APC gene as a result of mutations causing frameshifts into the second and third open reading frames.

Reference will be made to the following figures.

Figure 1a. Cytotoxic T cells from Balb/c mice that had been immunised with a vaccinia encoding the APC sequence in the second reading frame between codons 1418 and 1472, were obtained after stimulation for 5 days in vitro with the peptide sequence KYLKIKHLL. The target cells were L cells that had previously been transfected with the HLA gene H-2Db (called LDb). These cells were labelled with Cr51 and exposed to the treatments listed in the figure. They were then mixed with the effector cytotoxic T cells in the ratios shown in the figure (E/T ratio). The amount of killing was measured as % specific lysis as described in Townsend et al Cell Vol. 44, 959-968 1986. Note that infection with the vaccinia encoding the APC 2nd reading frame codons 1418-1472 resulted in killing by the T cells only after co-infection with the vaccinia virus encoding the HLA molecule K^d (K^dvac).

Figure 1b. A similar experiment was done using a colon carcinoma cell line as the target cell. The cell line is called LoVo (Brodsky et al Immunol. Rev 47, 3-61, 1979). This cell line lacks expression of beta-2 microglobulin, so to express HLA molecules in this cell, an additional vaccinia virus provided expression of beta-2 microglobulin (β2m) (see Yewdell et al J. Immunol, 152,

1163-1170, 1994). Note that the target cells were recognised by the cytotoxic T cells only when either i) the target cells were infected with beta-2 microglobulin vaccinia, and K^d vaccinia and the vaccinia encoding APC second reading frame codons 1418-1472, or ii) infected with Beta-2 microglobulin vaccinia and K^d vaccinia and then exposed to the peptide KYLKIKHLL.

Figure 2. Evidence for binding by the sequences QLKPSEKYL and KVLQMDFLV to HLA A2. The experiment was done as referred to in the text. Lane 1 shows the background level of A2 detected in the absence of peptide, and lane 2 the increase seen when a known binding peptide derived from influenza matrix protein was added to 20μm. In lanes 3-18 a set of peptides derived from the second and third reading frames of APC were added to 20μM. The sequences QLKPSEKYL (lane 11) and KVLQMDFLV (lane 18) markedly increased the level of A2 detected, indicating binding.

Figure 3. Evidence for binding by the sequence ILYYILPRK to HLA A3. The experiment was done as described for fig. 2. Lane 1 shows the level of A3 detected by the monoclonal antibody GAPA3 in the absence of peptide. Lanes 2 and 3 show a marked increased after addition of the peptide sequence ILYYILPRK to either 50μm (lane 2) or 5μm (lane 3). Lane 4 contained a known HLA A3 binding peptide that is known to be recognised by human A3 restricted cytotoxic T cells specific for HIV nef, as a positive control. Note in Lanes 7 and 8 that

the sequence QLKPSEKYL that was shown in Fig. 2 to bind HLA A2, did not bind HLA A3.

Figure 4 shows a Western Blot. Lane 1: a positive control extract from a colon cancer cell line SW480 (described in Smith et al., PNAS 90, 2846-2850, 1993) which expresses a mutant APC protein of approximately 147 kD which is close in size to the expected size of a recombinant form of mutant APC protein deriving from a recombinant vaccinia virus; Lanes 2-4: extracts from LK cells (5 x 10^5 cells/lane) infected with (20 p.f.u in 10^6 cells; 1.5 hours; 4 hours for recovery) three different subclones (7131c3 VAC, 7131c2 VAC, 7131c1 VAC) of a recombinant vaccinia virus encoding a full length mutant APC gene (7131 VAC); Lane 5: a positive control extract from a lymphoblastoid cell line derived from a patient with a mutation similar to that carried by the recombinant vaccinia virus 7131 VAC.

Figures 5(a)(b)(c) and (d) show the results for Cr^{51} release assays. In (a) and (b) the target cells are all murine L cells transfected with a gene encoding murine K^d Class I molecules (LKd cells). Lkd Un are Lkd cells not infected with vaccinia virus carrying any of the mutant APC gene. Lkd M-53-QVAC are Lkd cells infected with a recombinant vaccinia virus encoding a 53 amino acid fragment containing the KYLKIKHLL epitope. 7131/3 VAC are LKd cells infected with a recombinant vaccinia virus encoding the full length mutant APC protein from tumour 7131 and that contains the KYLKIKHLL epitope. NP147-55

are LKd cells treated with a peptide representing amino acids 147 to 155 from influenza nucleoprotein as a negative control. K-9-L are LKd cells treated with the peptide KYLKIKHLL which is present in the frameshifted APC protein sequence in tumour 7131. In (a) the lytotoxic T eymphocytes were murine and raised to the 9 residue peptide KYLKIKHLL. In (b) the cytotoxic T lymphocytes were murine and raised to the sequence NP147-155 of influenza virus. In (c) and (d) the two sets of cytotoxic T lymphocytes (against KYLKIKHLL and NP147-158 of influenza virus) were tested against L cells transfected with the histocompatibility molecule Db instead of Kd.

Part 1

Studies have shown (Myoshi et al., 1992 supra.), that the mutations in the APC gene tend to predominate between codons 1250-1550 (although many immunologically important frameshifts also occur elsewhere in the APC gene and these may also be investigated as described herein).

The predominance of mutations between codons 1250 and 1550 allows convenient screening for mutations by polymerise chain reaction (PCR) amplification of the APC DNA sequence between these codons, derived from tumour samples.

As an illustrative example of the procedure to be followed to identify useful immunogenic sequences, the

applicants have chosen to study frameshifts that read into (1) the 2nd reading frame lying between codons 1418 and 1472 that can arise by single base deletions or 2 base insertions and (2) the 2nd reading frame lying between codons 1472 and 1506, also arising by single base deletions or 2 base insertions. All numbering as used herein follows the APC sequence numbering as used in Miyoshi et al., 1992 supra.

DNA was extracted from tumour samples in accordance with standard techniques (eg as described in Sambrook, Fritsth, Maniatis (1989) Cold Spring Harbour Laboratory Press). For the tumour extractions, the nucleotide sequences lying between codons 1418 to 1472 and codons 1472 to 1506 were amplified by PCR in accordance with standard procedures using oligonucleotides A, B, C & D below:

```
A        Hind lll  Nco l
  5'CCC,AAG,CTT,A,CCATG,GCC,CCA,GTG,ATC,TTC,CAG,ATA,GC 3'

B                                   STOP Xho l Bgl ll
  3'CAC,CTG,GAT,TCG,TTC,GAC,GTC,ATT,GAG,CTC,TAG,AAG 5'

C        Hind lll Nco l
  5'CCC,AAGCTT,A,CCATG,GTG,CTG,CAG,TTC,AGA,GGG,TCC,AG 3'

D                                   STOP Xho l Bgl ll
  3'C,CTA,AAA,GAA,CAA,GTA,GGT,CGG,ACT,GAG,CTC,TAG,AAG 5'
```

Primers A and B were used to amplify the nucleotide sequences lying between codons 1418 to 1472 and primers C and D were used to amplify nucleotide sequences lying between codons 1472 to 1506. PCR was performed with

Stratagene P.F.U.. The following ingredients were mixed: (1) template DNA from tumour (titrated); (2) 10μls oligo's A and B or C and D from 4μ molar stock solutions; (3) 10μls x 10 Stratagene P.F.U. buffer; (4) 5μls GATC mix from 5m Molar stock solution; (5) H_2O to 100μls final volume. This mixture was heated to 95°C for 5 mins, then cooled to 80°C. Five units of Stratagene P.F.U. were then added. PCR was performed with 35 cycles consisting of 5 sec. at 92°C, 1 min. at 58°C and 1 min. at 72°C.

The oligonucleotides were designed to provide an ATG initiation codon contained within an NcoI restriction site at the start of the reading frames and a BglII restriction site at the end of the reading frames.

The amplified nucleotide sequences were digested with BglII and NcoI and cloned into the vaccinia expression vector PSC113OR.2 . PSC113OR.2 was derived from the vector pSC11 (Chakrabarti, S., et al Molec. Cell Biol. Vol. 5 No. 12 1985 p. 3402) by the insertion of an oligonucleotide into the SmaI site, as shown below.

Inserted sequence

6471(pSC11) Bam HI (SmaI) NcoI SmaI StuI
AAGTAGAATCATAAAGAACA GTGACGGATC CCACCATGG CCCGGGAAGG

BgIII
STOP STOP STOP (SmaI)Eco RI
CCTAGCTGAC TAGATCTGGG AATTCTGTGA GCGTATGGCA AACGAAGGAA

AAATTAGTTA TAGT AGCCGC ACTCGATGGGACATTTCAAC GTAAACCGTT

TAATAATATT TTGAATCTTA TTCCATTATC TGAAA

The oligonucleotide inserted into the SmaI site provides a kozac sequence upstream of an ATG initiation

site to allow expression of cloned open reading frames.

The amplified nucleotide sequences lying between codons 1418 to 1472 and codons 1472 to 1506 were sequenced and examined for encoding predictive amino acid sequences that were likely to bind to murine K^d Class I molecules on the basis of the nucleotide sequences encoding a Y residue at position 2 and L residue at position 9 (Falk et al Nature 351, 290-296, 1991). The nucleotide sequences encoding predictive polypeptide sequences considered as being likely to bind murine K^d Class I molecules and which were in the second frame (as determined from a comparison with the wild type sequence) were further investigated for their immunogenicity.

Nucleotide sequences encoding open reading frame from 1418 to 1472 in the second reading frame and considered as being likely to contain sequences that bind murine K^d Class I molecules were inserted into the vaccinia virus TK gene in accordance with standard procedures (eg Townsend et al., J. Exp. Med., Vol. 168, 1211-1224, 1988).

Mice were immunised i.v. with 10^7 plaque forming units (p.f.u) of recombinant vaccinia virus having a sequence insertion. At least 10 days later, spleen cells were removed. If a polypeptide is suitably immunogenic and therefore of potential use as a vaccine, it will induce a population of T cells carrying receptors specific for the particular polypeptide. Since T cells having the specific receptor will exist in low

concentration in the spleen (about 1 cell in 10,000), the population of specific T cells was expanded by exposure in vitro to the 9 residue peptide KYLKIKHLL (residues 1451-1459), using a method as described by Bastin, J., et al J. Exp. Med. Vol. 165, 1508-1523, 1987. 5-7 days later the growing T cells were either tested in a standard CR^{51} release assay for specific killing or re-stimulated and grown in IL-2 containing medium (also as discussed in Bastin et al 1987 supra.) and tested at later periods.

In the Cr^{51} release assay, the target cells which release Cr^{51} may, for example, be either murine L cells (Townsend et al Cell Vol. 44 959-968, 1986) infected with the recombinant vaccinia virus encoding the appropriate reading frame, or a cell line derived from human colon carcinoma similarly infected. To provide expression of appropriate MHC molecules in the target cells, they may be transfected with a gene coding the MHC molecule (Townsend et al 1986 supra.) or infected with a second vaccinia virus encoding the MHC molecule (Yewdell et al J. Immunol. Vol. 152 p. 1163-1170, 1994).

The results as shown in Fig. 1 indicate the following.

1. Mice immunised with recombinant vaccinia virus comprising a human colon carcinoma nucleotide sequence encoding predictive polypeptide sequences in the second reading frame and considered as likely to bind murine K^d Class I molecules had cytotoxic T

cells which recognised target cells exposed to the polypeptide sequence (eg by infection with a recombinant vaccinia virus encoding the peptide) specifically when murine MHC Class I K^d molecules are also expressed in the target cell.

2. The cytotoxic T cells also recognised both types of target cells coinfected with the vaccinia virus encoding the complete open reading frame (1418-1472) and the vaccinia virus encoding the murine MHC K^d molecule. This demonstrates (a) that the 2nd reading frame lying between codons 1418-1472 was immunogenic to Balb/c mice, (b) that the epitope 1451-1459 could be presented from within the longer fragment 1418-1472.

These results support the present applicants' teaching that the proteins encoded by the 2nd and 3rd reading frames and polypeptide/peptide fragments thereof of a gene, such as the APC gene, can be immunogenic in mammals.

As the sequence of murine APC is closely related to the human APC sequence, the result also establishes that mice are not immunogenically tolerant to the proteins encoded in the 2nd and 3rd reading frames of human APC.

Part 2

The experiments described in Part 1 utilised a recombinant vaccinia virus having as a sequence

insertion, the open reading frame from 1418 to 1472 in the second reading frame and considered as likely to contain sequences that bind murine K^d Class I molecules. The experiments in Part 2 make use of a recombinant vaccinia virus having as a sequence insertion a full length mutant APC gene. The first objective was to demonstrate that a full length mutant APC protein would be digested as expected in cells to produce a range of peptide fragments which originate from the polypeptide encoded by the nucleotide sequence downstream of the frameshift mutation. This demonstration required the construction of full length mutant APC coding sequence. Given below are the sequences of the oligonucleotides E and F used to amplify by PCR the fragment comprising mutant sequence from tumour 7131 and the complete sequence of the amplified fragment with the Psp 14061 restriction site shown and the 2 base insertion shown in bold (AA) at codon 1438. The principal was to include in the amplified sequence a restriction enzyme site Psp 14061 that is unique to the APC gene. This site can then be used to recombine the normal left hand fragment (5' fragment) of the normal gene with the mutant right hand fragment (3' fragment) amplified from the tumour sample using standard technology as described in Sambrook et al 1989 supra.,. The complete mutant cDNA can then be cleaved out with BamHI and XhoI to subclone into pSC65 for making the recombinant vaccinia by standard procedures. DNA was extracted from a tumour sample

(designated 7131) in accordance with standard techniques (Sambrook et al., 1989 supra). The tumour 7131 has a 2 base pair insertion into codon 1438 of the APC gene. A short mutant fragment from within exon 15 was cloned as follows. The nucleotide sequence comprising the fragment was amplified by PCR in accordance with standard procedures using oligonucleotides E & F below for amplifying the mutant fragment in tumour 7131 from the PSP 14061 restriction enzyme site to the end of the mutant reading frame A-53-Q and reconstructing the complete mutant APC gene with the wild type BamHI Psp14061 fragment.

E

BamHI NcoI (3440) Psp 14061

5' CGGGATCC,A,CC,ATG,GCC,AAT,TAT,AGT,GAA,CGT,TAC,TCT,GAA,G 3'

F

STOP XhoI BglII

3' CAC,CTG,GAT,TCG,TTC,GAC,GTC,ATT,GAG,CTC,TAG,AAG, 5'

(4435)

Below is given the sequence across the Psp 14061 site amplified with oligos E and F. The product should be 955 bp.

Psp1406I

```
                ACCAATTATAGTGAACGTTACTCTGAAGAAGAACAGCATGAAGAAGAAGAGAGACCAACA
3439 -+---------+---------+---------+---------+---------+---------+-------- 3498
                TGGTTAATATCACTTGCAATGAGACTTCTTCTTGTCGTACTTCTTCTTCTCTCTGGTTGT

                T  N  Y  S  E  R  Y  S  E  E  E  Q  H  E  E  E  E  R  P  T

                AATTATAGCATAAAATATAATGAAGAGAAACGTCATGTGGATCAGCCTATTGATTATAGT
3499 -+---------+---------+---------+---------+---------+---------+-------- 3558
                TTAATATCGTATTTTATATTACTTCTCTTTGCAGTACACCTAGTCGGATAACTAATATCA

                N  Y  S  I  K  Y  N  E  E  K  R  H  V  D  Q  P  I  D  Y  S

                TTAAAATATGCCACAGATATTCCTTCATCACAGAAACAGTCATTTTCATTCTCAAAGAGT
3559 -+---------+---------+---------+---------+---------+---------+-------- 3618
                AATTTTATACGGTGTCTATAAGGAAGTAGTGTCTTTGTCAGTAAAAGTAAGAGTTTCTCA

                L  K  Y  A  T  D  I  P  S  S  Q  K  Q  S  F  S  F  S  K  S

                TCATCTGGACAAAGCAGTAAAACCGAACATATGTCTTCAAGCAGTGAGAATACGTCCACA
3619 -+---------+---------+---------+---------+---------+---------+-------- 3678
                AGTAGACCTGTTTCGTCATTTTGGCTTGTATACAGAAGTTCGTCACTCTTATGCAGGTGT

                S  S  G  Q  S  S  K  T  E  H  M  S  S  S  S  E  N  T  S  T

                CCTTCATCTAATGCCAAGAGGCAGAATCAGCTCCATCCAAGTTCTGCACAGAGTAGAAGT
3679 -+---------+---------+---------+---------+---------+---------+-------- 3738
                GGAAGTAGATTACGGTTCTCCGTCTTAGTCGAGGTAGGTTCAAGACGTGTCTCATCTTCA

                P  S  S  N  A  K  R  Q  N  Q  L  H  P  S  S  A  Q  S  R  S

MCR REGION 5'
                GGTCAGCCTCAAAAGGCTGCCACTTGCAAAGTTTCTTCTATTAACCAAGAAACAATACAG
3739 -+---------+---------+---------+---------+---------+---------+-------- 3798
                CCAGTCGGAGTTTTCCGACGGTGAACGTTTCAAAGAAGATAATTGGTTCTTTGTTATGTC

                G  Q  P  Q  K  A  A  T  C  K  V  S  S  I  N  Q  E  T  I  Q

                ACTTATTGTGTAGAAGATACTCCAATATGTTTTTCAAGATGTAGTTCATTATCATCTTTG
3799 -+---------+---------+---------+---------+---------+---------+-------- 3858
                TGAATAACACATCTTCTATGAGGTTATACAAAAAGTTCTACATCAAGTAATAGTAGAAAC

                T  Y  C  V  E  D  T  P  I  C  F  S  R  C  S  S  L  S  S  L

                TCATCAGCTGAAGATGAAATAGGATGTAATCAGACGACACAGGAAGCAGATTCTGCTAAT
3859 -+---------+---------+---------+---------+---------+---------+-------- 3918
                AGTAGTCGACTTCTACTTTATCCTACATTAGTCTGCTGTGTCCTTCGTCTAAGACGATTA

                S  S  A  E  D  E  I  G  C  N  Q  T  T  Q  E  A  D  S  A  N
```

```
                ACCCTGCAAATAGCAGAAATAAAAGAAAAGATTGGAACTAGGTCAGCTGAAGATCCTGTG
3919            -+---------+---------+---------+---------+---------+--------  3978
                TGGGACGTTTATCGTCTTTATTTTCTTTTCTAACCTTGATCCAGTCGACTTCTAGGACAC

                T  L  Q  I  A  E  I  K  E  K  I  G  T  R  S  A  E  D  P  V  -

                AGCGAAGTTCCAGCAGTGTCACAGCACCCTAGAACCAAATCCAGCAGACTGCAGGGTTCT
3979            -+---------+---------+---------+---------+---------+--------  4038
                TCGCTTCAAGGTCGTCACAGTGTCGTGGGATCTTGGTTTAGGTCGTCTGACGTCCCAAGA

                S  E  V  P  A  V  S  Q  H  P  R  T  K  S  S  R  L  Q  C  S  -

                AGTTTATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTTCAGGAGCGAAATCT
4039            -+---------+---------+---------+---------+---------+--------  4098
                TCAAATAGAAGTCTTAGTCGGTCCGTGTTTCGACAACTTAAAAGAAGTCCTCGCTTTAGA

                S  L  S  S  E  S  A  R  H  K  A  V  E  F  S  S  G  A  K  S  -

                CCCTCCAAAAGTGGTGCTCAGACACCCAAAAGTCCACCTGAACACTATGTTCAGGAGACC
4099            -+---------+---------+---------+---------+---------+--------  4158
                GGGAGGTTTTCACCACGAGTCTGTGGGTTTTCAGGTGGACTTGTGATACAAGTCCTCTGG

                P  S  K  S  G  A  Q  T  P  K  S  P  P  E  H  Y  V  Q  E  T

                CCACTCATGTTTAGCAGATGTACTTCTGTCAGTTCACTTGATAGTTTTGAGAGTCGTTCG
4159            -+---------+---------+---------+---------+---------+--------  4218
                GGTGAGTACAAATCGTCTACATGAAGACAGTCAAGTGAACTATCAAAACTCTCAGCAAGC

                P  L  M  F  S  R  C  T  S  V  S  S  L  D  S  P  E  S  R  S  -

                ATTGCCAGCTCCGTTCAGAGTGAACCATGCAGTGGAATGGTAAGTGGCATTATAAGCCCC
4219            -+---------+---------+---------+---------+---------+--------  4278
                TAACGGTCGAGGCAAGTCTCACTTGGTACGTCACCTTACCATTCACCGTAATATTCGGGG

                I  A  S  S  V  Q  S  E  P  C  S  G  M  V  S  G  I  I  S  P  -

                                                          Codon 1438

                AGTGATCTTCCAGATAGCCCTGGACAAACCATGCCACCAAGCAGAAGTAAAAAACACCTCCA
4279            -+---------+---------+---------+---------+---------+--------  4338
                TCACTAGAAGGTCTATCGGGACCTGTTTGGTACGGTGGTTCGTCTTCATTTTTTGTGGAGGT

                S  D  L  P  D  S  P  G  Q  T  M  P  P  S  R  S  K  K  H  L  H  -

                CCACCTCCTCAAACAGCTCAAACCAAGCGAGAAGTACCTAAAAATAAAGCACCTACTGCT
4339            -+---------+---------+---------+---------+---------+--------  4398
                GTGGAGGAGTTTGTCGAGTTTGGTTCGCTCTTCATGGATTTTTATTTCGTGGATGACGA

                H  L  L  K  Q  L  K  P  S  E  K  Y  L  K  I  K  H  L  L  L  -
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     GAAAAGAGAGAGAGTGGACCTAAGCAAGCTGCAGTAA
4399 -+---------+---------+---------+----- 4435
     CTTTTCTCTCTCTCACCTGGATTCGTTCGACGTCATT

b     K  R  E  R  V  D  L  S  K  L  O  *
```

PCR was performed at optimum titrations with Stratagene P.F.U. The following ingredients were mixed: (1) template DNA from tumour (titrated); (2) 10 μls oligo's E and F from 4μ molar stock solutions; (3) 10 μls x 10 Stratagene P.F.U buffer; (4) 5 μls GATC mix from 5 m molar stock solution; (5) H_2O to 100 μls final volume. This mixture was heated to 95°C for 5 mins, then cooled to 80°C. Five units of Stratagene P.F.U were then added. PCR was performed with 35 cycles consisting of 5 sec. at 92°C, 1 min at 58°C and 1 min. at 72°C.

The amplified fragment was then recombined with a full length wild type APC cDNA followed by cloning into the vaccinia expression vector PSC65 (a gift from Dr. Moss of the National Institute of Health, Washington US: this vector is similar to PSC1130R.2 which could be used as an alternative vector) and sequencing as described in Part 1. Recombinant vaccinia encoding the full length mutant APC cDNA were then produced as described in Part 1.

L cells were then infected (20 p.f.u in 10^6 cells; 1.5 hours; 4 hours for expression) with recombinant vaccinia (subclones 7131c3 VAC, 7131c2 VAC and 7131c1 VAC) and the size of proteins expressed determined by Western blot analysis (5 x 10^5 cells/lane) using the antibody FE9 (available from Oncogene Science Inc., 106 Charles Lindbergh Blvd, Uniondale, NY 11553-3649, USA) which reacts with the non-mutant N-terminal region of the protein as described by Smith et al., PNAS 90, 2846-2850 (1995).

The recombinant vaccinias were shown to express a mutant APC protein of the expected size and the blot is shown in Fig. 4. Lane 1 is a positive control extract from a colon cancer cell line SW480 (described in Smith et al., PNAS 1993 supra) which expresses a mutant APC protein of approximately 147 kD which is close in size to the expected size of a recombinant form of mutant APC protein (approximately 165 kD) deriving from the recombinant vaccinias. Lanes 2 to 4 are extracts from L cells infected with three different subclones (7131c3 VAC, 7131c2 VAC and 7131c1 VAC) of vaccinia. A major band is seen running just above the SW480 band and close in size to the band in lane 5 from SM501 another positive control derived from a lymphoblastoid cell line from a patient who has inherited a mutation similar to that carried by the recombinant vaccinias.

The results confirm that the recombinant vaccinias carry a full length mutant APC gene and further that they are able to express a mutant APC polypeptide of expected size. Lanes 2 to 4 also show bands representing a series of proteins with mws of less than 147 kD and which also react with the FE9 antibody. These probably result from digestion of the full length mutant APC gene in the L cells.

Experiments were also conducted to investigate the digestion of the full length mutant form of the APC protein and presentation of a fragment thereof for recognition by cytotoxic T lymphocytes.

In Fig 5(a) cytotoxic T lymphocytes were raised in Balb/c mice that had been immunised as earlier described with a recombinant vaccinia virus having as an insertion the 9 residue peptide KYLKIKHLL (residues 1451-1459) identified in Part 1. The 9 residue peptide KYLKIKLL is derived from the commonest frameshift antigen (present in tumour 7131) and is recognised by murine cytotoxic T lymphocytes in association with the K^d Class I molecule. These cytotoxic T lymphocytes were tested in a standard CR^{51} release array using as target cells (i) LKd Un which are murine L cells transfected with a gene encoding murine K^d Class I molecules but not infected with vaccinia virus carrying any of the mutant APC gene; (ii) Lkd M-53-QVAC which are murine L cells transfected with a gene encoding murine K^d class 1 molecules and a recombiant vaccinia virus encoding a 53 amino acid fringement containing the KYLKIKHLL epitope as a positive control; (iii) 7131/3VAC which are murine L cells transfected with a gene encoding murine K^d Class I molecules and a recombinant vaccinia virus encoding the full length mutant APC protein from tumour 7131 and that contains the KYLKIKHLL epitope; (iv) NP147-155 which are murine L cells transfected with a gene encoding murine K^d Class 1 molecules and treated with a peptide representing amino acids 147 to 155 from influenza nucleoprotein as a negative control; and (v) K-9-L which are murine L cells transfected with a gene encoding murine K^d Class I molecules and treated with the peptide KYLKIKHLL which is

present in the frameshifted APC protein sequence in tumour 7131.

The figure shows that the cells infected with a recombinant vaccinia virus encoding the full length mutant APC gene from tumour 7131 were recognised by cytotoxic T lymphocytes specific to the 9 residue peptide KYLKIKHLL.

In Fig 5(b) the same set of target cells were tested with cytotoxic T lymphocytes raised in Balb/c mice that had been immunised with the sequence NP 147-155 of influenza virus. The results show that cells infected with a recombinant vaccinia virus encoding the full length mutant APC gene from tumour 7131 are recognized only by cytotoxic T lymphocytes specific to the APC frameshift antigens and not by other cytotoxic T lymphocytes that recognize other antigens in association with murine K^d Class I molecules.

In Figs 5(c) and 5(d) the two sets of cytotoxic T lymphocytes (against KYLKIKHLL in (c) and against NP 147-155 of influenza virus in (d)) were tested against L cells transfected with the histocompatibility molecule Db instead of Kd. The results show that the cytotoxic T lymphocytes will only recognize their antigen when presented in the context of the correct histocompatibility molecule.

In conclusion, the experiments reported above the results for which are given in Figs 5(a)(b)(c)(d), demonstrate that the full length mutant form of APC

protein found in tumour 7131, that represents the commonest class of mutation found in naturally occurring tumours (Townsend et al Nature 371, 662 (1994)) can be digested inside cells to produce the peptide epitope KYLKIKHLL from within the frameshift sequence in a form that can be recognized by cytotoxic T lymphocytes at the cell surface.

Part 3

In relation to the preparation of a vaccine it may be useful to identify particular epitopes within the APC open reading frames which are immunogenic and which may be used either alone or in combination with other such sequences in a vaccine preparation. Falk et al (Nature 361, 290-296, 1991) established that peptides with certain identifiable features (such as Y at position 2 and L at position 9) will bind to particular MHC molecules with higher probability than sequences not having the particular features. The present applicants have demonstrated (Elvin, J., et al., 1993 supra.) that the sequences defined by Falk et al may bind human MHC Class I molecules with low or high affinity. To identify peptides which bind with either high or low affinity, the applicants have developed an assay for binding of MHC molecules based on their observations that peptides stabilise the structure of MHC molecules when bound (Townsend, Al., et al., Nature Nol. 340 No. 6233 p443 1989). This assay has been used to define epitopes of

malaria for instance (Hill et al Nature Vol. 360, 434, 1992).

The present applicants have applied this assay to the new reading frames of the APC gene resulting from frameshifts and identified several peptides that bind the common human MHC molecules A2 and A3. Figures 2 and 3 provide evidence for the sequence QLKPSEKYL (codons 1445-1453, 2nd reading frame) binding the A2 molecule and the sequence KVLQMDFLV (codons 1494-1502 in the second reading frame, ILYYILPRK (codons 1486-1495, 2nd reading frame) binding the A3 molecule. In addition, the second reading frame sequence lying between codons 1418 and 1472 which is immunogenic to Balb/c mice, also contains a sequence motif (Y at position 2 and L at position 9) appropriate for binding the MHC A24 molecule, the commonest allele in Japan where colorectal carcinoma is particularly prevalent.

In order to finally establish that a particular sequence deriving from a new open reading frame resulting from a frameshift is immunogenic in man, volunteers may be immunised with prototype vaccines containing or able to express in vivo the peptides encoded in the 2nd and 3rd reading frames of a gene such as APC.

Relating to the preparation of vaccines for human use that stimulate CTL efficiently, the applicants will investigate in accordance with standard procedures and methodologies the utility of preparations including recombinant vaccinia virus or other equivalent viral

vectors, recombinant Ty particles and various adjuvants that are claimed to stimulate CTL. Such trials will be for the purpose of

(a) establishing the immunogenicity of vaccines; and

(b) identifying new epitopes

Further studies in patients will be of two kinds:

(a) vaccinations of individuals with well defined epitopes in their tumours with secondary deposits that could be monitored for T cell invasion; and

(b) vaccination of patients with frameshifts using their frameshift protein.

This type of trial would be double-blinded and prospective, the vaccine being given as an adjuvant therapy after surgery for the presenting primary tumour.

CLAIMS

1. A pharmaceutical which comprises either part or all of a polypeptide or a nucleotide sequence coding for said part or all of a polypeptide, which polypeptide is substantially homologous to a mutant protein produced in consequence of a mutation in a gene sequence which shifts translation from the gene sequence out of the first reading frame into either the second or third reading frames downstream of the mutation and which said part or all of a polypeptide is able to invoke an immune response against the mutant protein.

2. A pharmaceutical according to claim 1 wherein the mutation in the gene sequence is associated with a cancer.

3. A pharmaceutical according to claim 2 wherein the cancer is a carcinoma.

4. A pharmaceutical according to claim 3 wherein the carcinoma is a gastro-intestinal carcinoma.

5. A pharmaceutical according to any one of claims 1 to 4 wherein the gene sequence is that of the adenomatous polyposis coli (APC) gene of chromosome 5q21.

6. A pharmaceutical according to claim 5 wherein the mutation is between codons 1418 and 1472 of the APC

gene.

7. A pharmaceutical according to claim 5 wherein the mutation is between codons 1472 and 1506 of the APC gene.

8. A pharmaceutical according to claim 5 which comprises either a peptide selected from

KYLKIKHLL

QLKPSEKYL

ILYYILPRK

KVLQMDFLV

or a nucleotide sequence coding therefor.

9. A pharmaceutical according to any one of claims 1 to 8 which is formulated as a vaccine.

10. A pharmaceutical according to claim 9 wherein the vaccine comprises an adjuvant.

11. A pharmaceutical according to claim 9 or claim 10 wherein the vaccine comprises a viral vector.

12. A pharmaceutical according to claim 11 wherein the viral vector is based upon a pox virus.

13. A pharmaceutical according to claim 12 wherein the pox virus is vaccinia virus.

14. A pharmaceutical according to claim 9 or claim 10 wherein the vaccine comprises recombinant Ty particles.

15. A pharmaceutical according to any one of claims 9 to 14 wherein the vaccine is a therapeutic vaccine.

16. A pharmaceutical according to any one of claims 9 to 14 wherein the vaccine is a prophylactic vaccine.

17. A pharmaceutical according to any one of claims 1 to 16 wherein the immune response is cell-mediated.

18. A nucleotide sequence which codes for a said part or all of a polypeptide as defined by claim 1.

19. A transfer, expression or vaccine vector containing a nucleotide sequence according to claim 18.

20. A host cell comprising a vector according to claim 19.

21. Use of part or all of a polypeptide or a nucleotide sequence coding for said part or all of a polypeptide, which polypeptide is substantially homologous to a mutant protein produced in consequence of a mutation in a gene sequence which shifts translation from the gene sequence out of the first reading frame into either the second or third reading frames downstream of the mutation and which said part or all of a polypeptide is able to involve an immune response against the mutant protein, in the preparation of a medicament for therapeutic or prophylactic vaccination against a disease state in a mammalian subject which is associated with the frameshifting mutation.

22. A method which comprises administering to a patient a pharmaceutical according to any one of claims 1 to 17 in order to invoke an immune response against the mutant protein.

Fig.1(a).

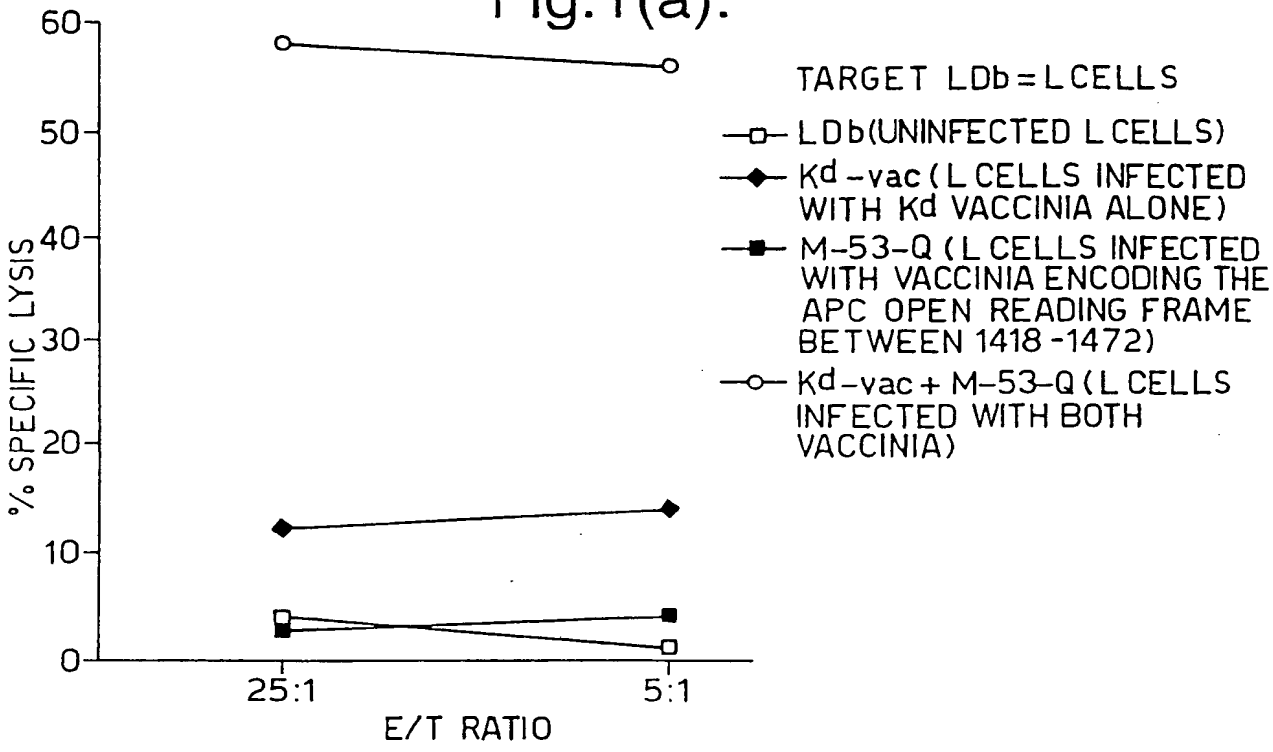

60
50
40
30
20
10
0
% SPECIFIC LYSIS
25:1
5:1
E/T RATIO
TARGET LDb=L CELLS
LDb(UNINFECTED L CELLS)
Kd-vac(L CELLS INFECTED WITH Kd VACCINIA ALONE)
M-53-Q(L CELLS INFECTED WITH VACCINIA ENCODING THE APC OPEN READING FRAME BETWEEN 1418-1472)
Kd-vac+M-53-Q(L CELLS INFECTED WITH BOTH VACCINIA)

Fig.1(b).

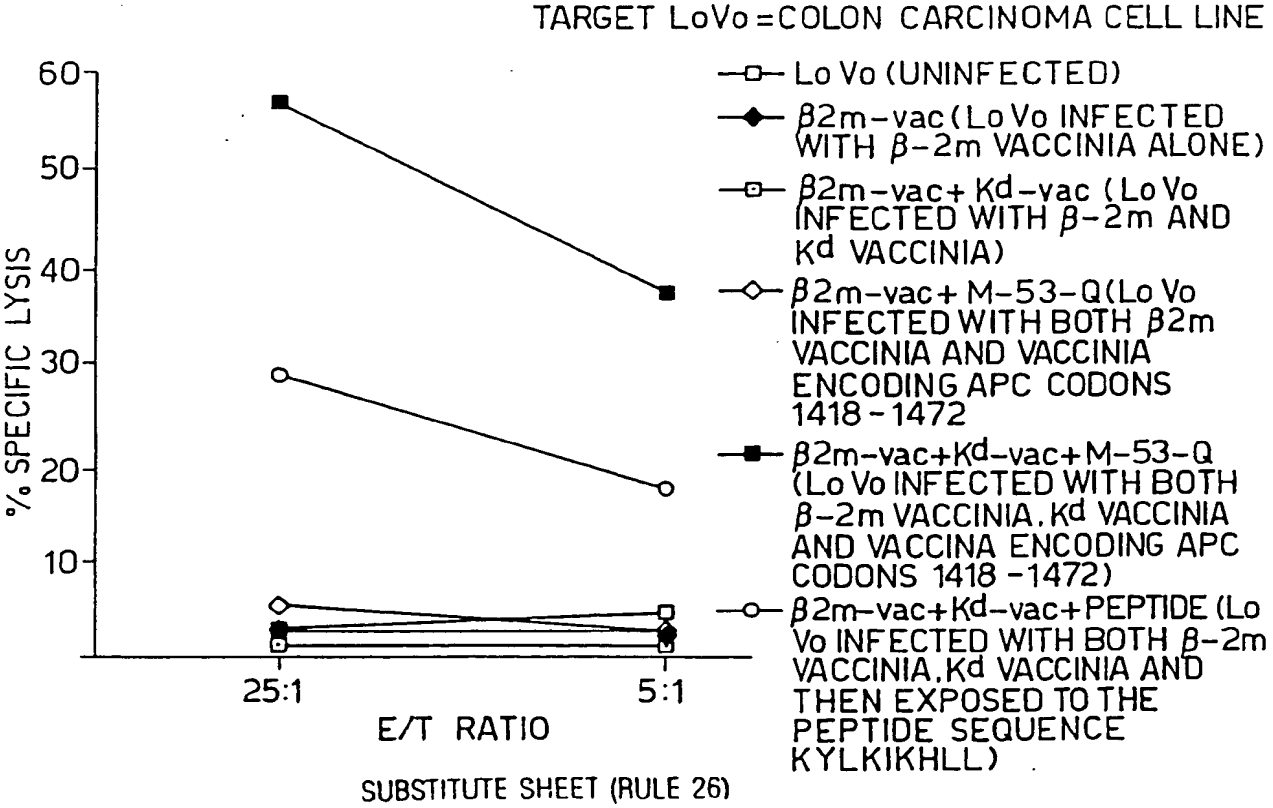

TARGET LoVo=COLON CARCINOMA CELL LINE
60
50
40
30
20
10
% SPECIFIC LYSIS
25:1
5:1
E/T RATIO
Lo Vo (UNINFECTED)
β2m-vac(Lo Vo INFECTED WITH β-2m VACCINIA ALONE)
β2m-vac+Kd-vac (Lo Vo INFECTED WITH β-2m AND Kd VACCINIA)
β2m-vac+M-53-Q(Lo Vo INFECTED WITH BOTH β2m VACCINIA AND VACCINIA ENCODING APC CODONS 1418-1472
β2m-vac+Kd-vac+M-53-Q (Lo Vo INFECTED WITH BOTH β-2m VACCINIA, Kd VACCINIA AND VACCINA ENCODING APC CODONS 1418-1472)
β2m-vac+Kd-vac+PEPTIDE (Lo Vo INFECTED WITH BOTH β-2m VACCINIA, Kd VACCINIA AND THEN EXPOSED TO THE PEPTIDE SEQUENCE KYLKIKHLL)

Fig.2.

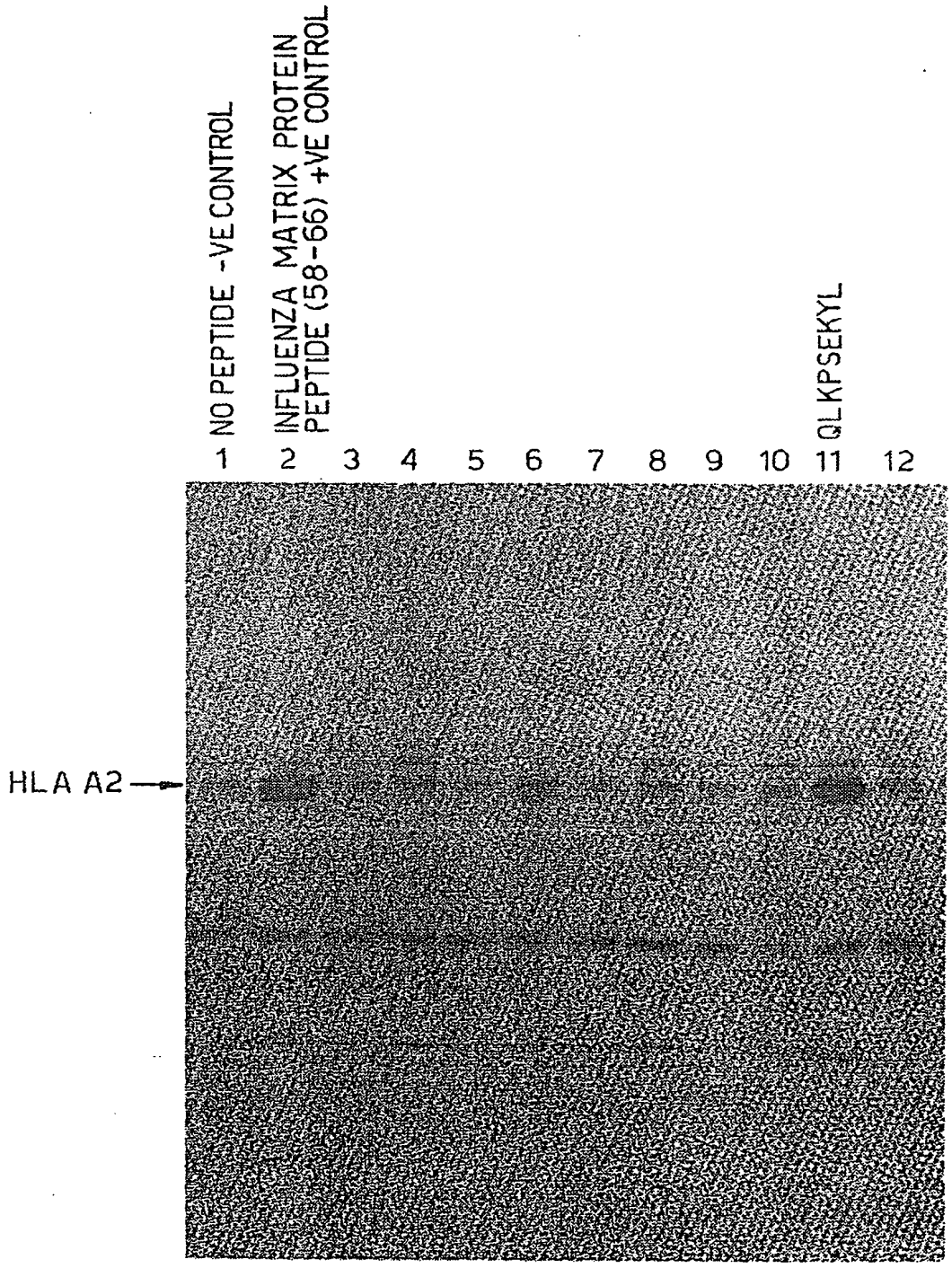

NO PEPTIDE -VE CONTROL
INFLUENZA MATRIX PROTEIN PEPTIDE (58-66) +VE CONTROL
QLKPSEKYL
1 2 3 4 5 6 7 8 9 10 11 12
HLA A2

Fig.2 (Cont).

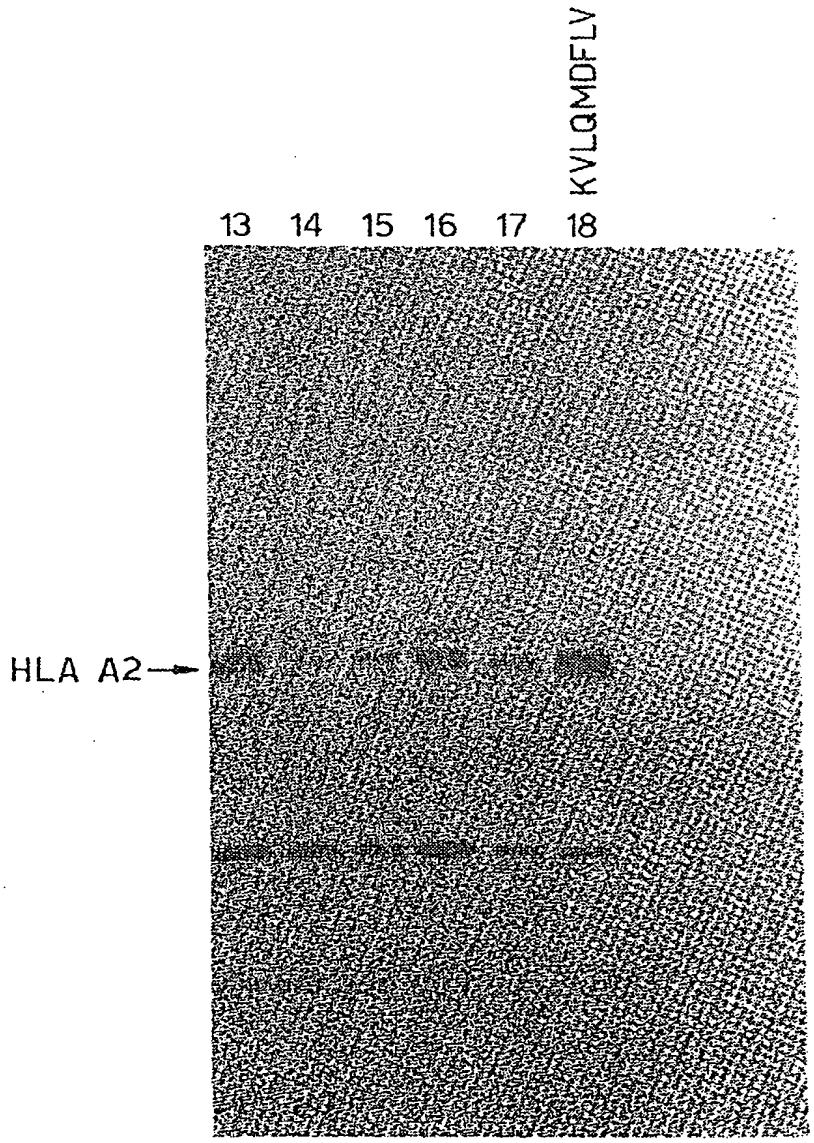


Fig.3.

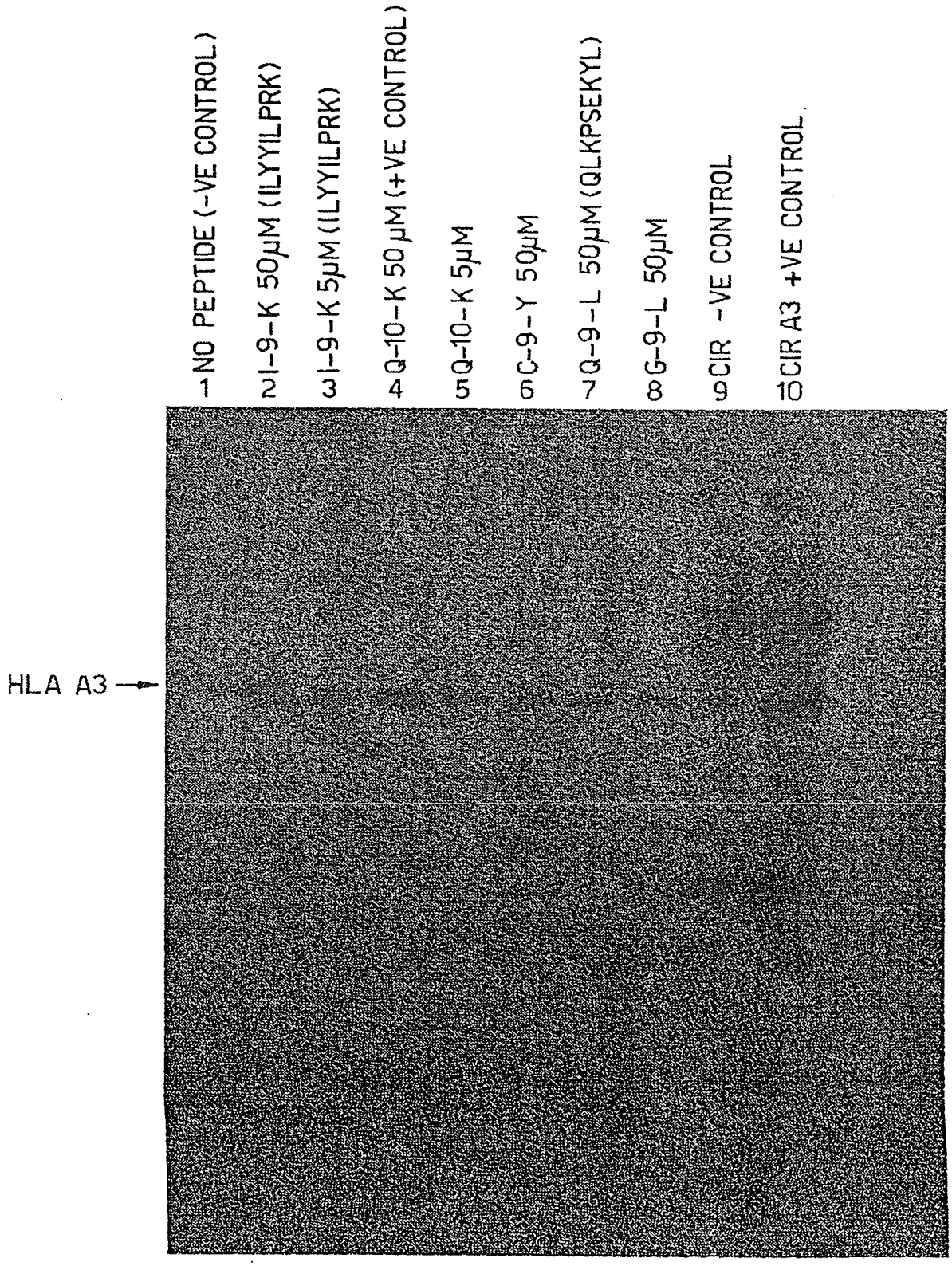
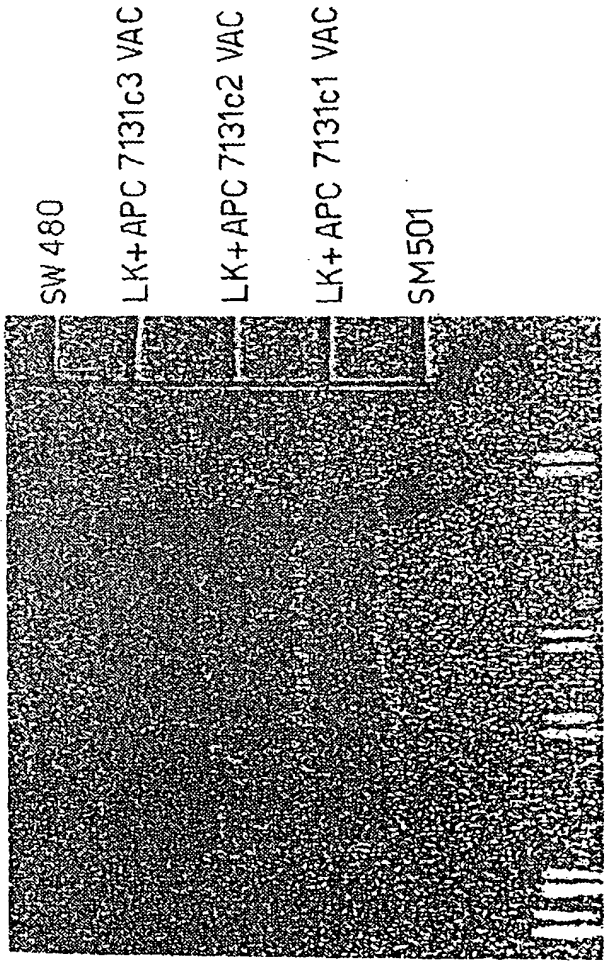
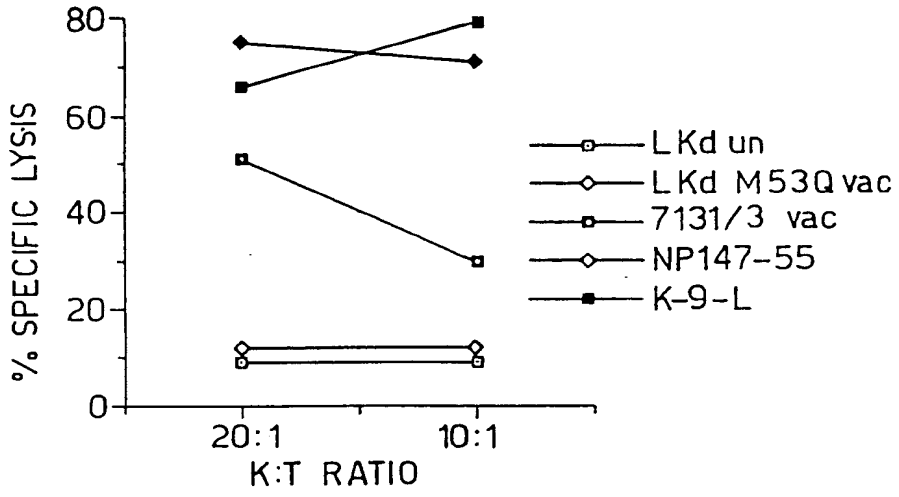

1 NO PEPTIDE (-VE CONTROL)
2 I-9-K 50μM (ILYYILPRK)
3 I-9-K 5μM (ILYYILPRK)
4 Q-10-K 50μM (+VE CONTROL)
5 Q-10-K 5μM
6 C-9-Y 50μM
7 Q-9-L 50μM (QLKPSEKYL)
8 G-9-L 50μM
9 CIR -VE CONTROL
10 CIR A3 +VE CONTROL
HLA A3

Fig.4.



1. ANTIBODY FE9
2. 2.5×10^5 CELLS/LANE
3. 20 p.f.u IN 10^6 CELLS

Fig.5(a).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60
40
20
0
% SPECIFIC LYSIS
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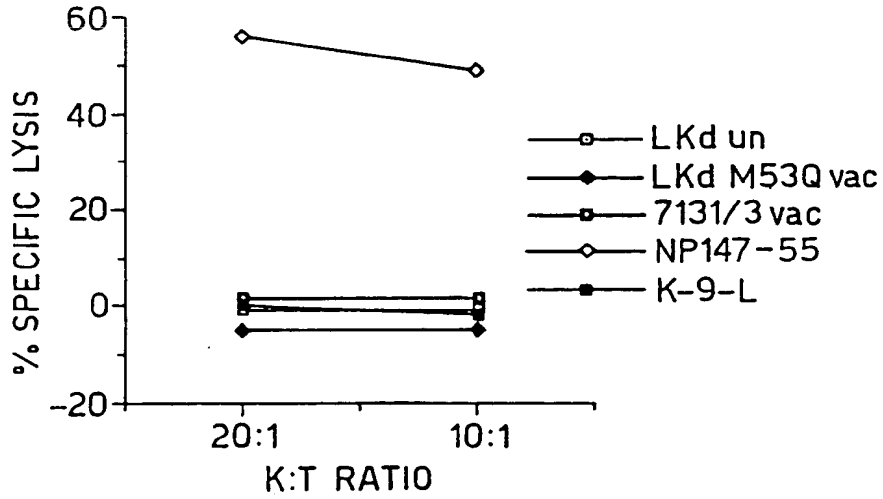
10:1
K:T RATIO
LKd un
LKd M53Q vac
7131/3 vac
NP147-55
K-9-L

Fig.5(b).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40
20
0
-20
% SPECIFIC LYSIS
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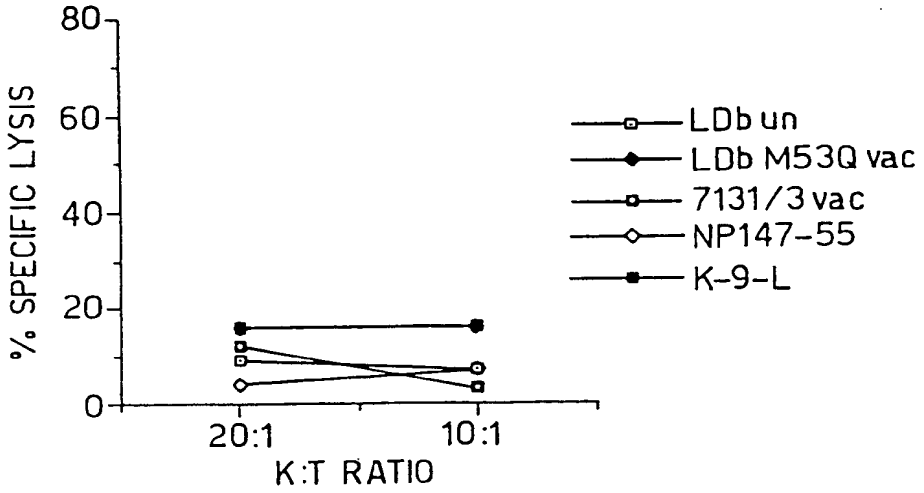
10:1
K:T RATIO
LKd un
LKd M53Q vac
7131/3 vac
NP147-55
K-9-L

Fig.5(c).


80
60
40
20
0
% SPECIFIC LYSIS
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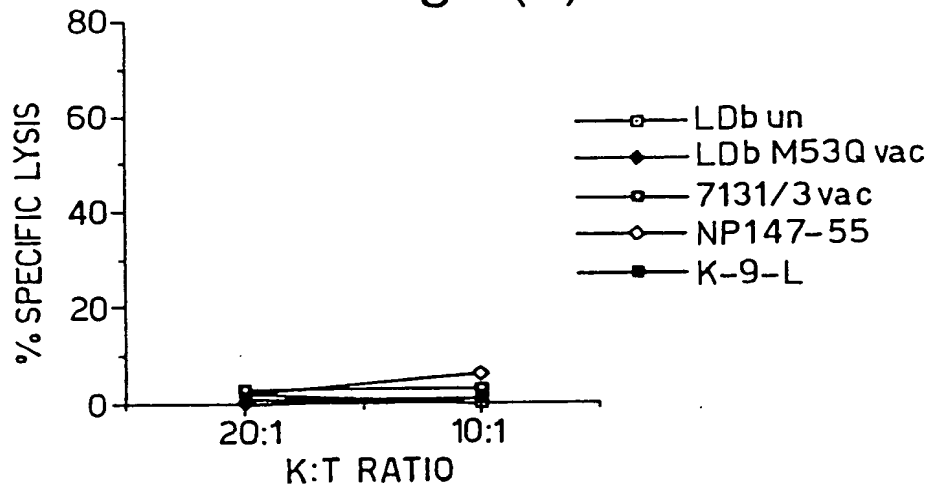
10:1
K:T RATIO
LDb un
LDb M53Q vac
7131/3 vac
NP147-55
K-9-L

Fig.5(d).


80
60
40
20
0
% SPECIFIC LYSIS
20:1
10:1
K:T RATIO
LDb un
LDb M53Q vac
7131/3 vac
NP147-55
K-9-L